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Molecular Epidemiology of Cytomegalovirus Infection in Kindergarten Children

Chen-Yang Shen, Bao-Li Chang, Shu-Fen Chang, Show-Lin Yang, Su-Ling Tseng, Chi-Yu Chen, and Cheng-Wen Wu

Institute of Biomedical Sciences, Academia Sinica, Taipei, Taiwan

This study was conducted to determine the source of cytomegalovirus (CMV) excretion in children who had persistent viruria. A total of 287 children, ages 3–5 years, enrolled in two kindergartens were followed for 9 months, and 28.8% of 139 CMV-infected children were found to have shed virus persistently in urine for ≥3 months. A polymerase chain reaction (PCR)-based method provided direct evidence to demonstrate differences in CMV strains shed by 32 persistent shedders selected at random. The glycoprotein B (gB) nucleotide regions of CMV were amplified and analyzed by restriction enzyme digestion. The CMV strains shed by most of these children showed different restriction profiles compared with strains from their playmates. It is considered that persistent reactivation of endogenous CMV is the most plausible explanation.

KEY WORDS: epidemiology, viral recurrence

INTRODUCTION

Cytomegalovirus (CMV), a member of the herpesvirus group, causes widespread infection but rarely kills its host under normal conditions [reviewed by Grundy, 1990]. After infection, CMV is not eliminated from the host. Instead, the virus can escape from the host's immune response and can either remain in a latent state, which reactivates intermittently, or persist with low-grade shedding. These conditions allow further transmission of the virus to new hosts. Therefore, crowded settings such as day care centers or kindergartens have been suggested frequently to pose a high risk for CMV acquisition and transmission. This possibility has been enhanced by demonstrating the similarity of restriction endonuclease digestion patterns of CMV DNA isolated from children attending day care centers [Adler, 1985].

Persistent subclinical shedding in urine is common in healthy young children in kindergartens [Shen et al., 1993b]. During the 1-year follow-up period of the study, most CMV-seropositive children in a kindergarten persistently or intermittently shed CMV in their urine. CMV recurrence in these infected children may be due to reactivation of endogenous CMV or, as is generally thought, to reinfection with exogenous CMV sources from playmates or family members [Alder, 1985]. However, indirect evidence, including the absence of anti-CMV IgM antibodies among infected children, the absence of virus in most of their family members, and no seroconversion among seronegative children during follow-up, does not favor the possibility of reinfection with exogenous CMV [Shen et al., 1993b]. We therefore question whether the idea that frequent transmission of CMV occurs among young children in kindergartens or day care centers does, in fact, apply to all child populations. The present study was undertaken to define the source of persistent CMV shedding in kindergarten children with subclinical infection and to provide direct evidence to demonstrate the similarities or differences in CMV strains shed by individual children.

MATERIALS AND METHODS

A total of 287 children, ages 3–5 years, enrolled in two kindergartens in Taipei were followed for 9 months (October 1993–July 1994). Informed consent was obtained from parents of the study subjects. Urine specimens (3–5 ml) were collected from the children every 3 weeks to 1 month, and the urine was inoculated onto human embryonic lung cell (HEL) cultures. CMV was detected by its characteristic cytopathic effect. When cytopathology involved at least 80% of the cell monolayer, the following strategy was used: First, a polymerase chain reaction (PCR)-dot hybridization procedure [Shen et al., 1994] using a primer pair and probe derived from the immediate-early gene (IE) gene was employed to confirm the cytopathology. Second, different categories of urinary CMV shedding were defined on the basis of individual shedding profiles. These categories were: persistent infection, i.e., children who shed CMV continuously for ≥3 months; intermittent infection, i.e., children who shed virus in urine, but the period of continuous shedding was <3 months; seropositive nonshedding, i.e., anti-CMV IgG seropositive chil-

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Address reprint requests to: Dr. Chen-Yang Shen, Institute of Biomedical Sciences, Academia Sinica, Taipei 11529, Taiwan.

dren who did not shed CMV on any occasion during follow-up. Third, DNA was extracted from the cell-free medium of each infected HEL culture that showed the cytopathic effect according to the method described by Hirt [1967]. The major interest was to define the infection source in children and it focused on CMV shed persistently and concurrently by individual children. Therefore, 32 CMV isolates were selected at random from persistent shedders at one time point (the specimens were collected in the 6th month of follow-up). Fourth, a PCR-based method [Chou and Dennison, 1991] to differentiate CMV strains by identifying the nucleotide sequence variations in the envelope glycoprotein B (gB) gene of CMV was used to carry out primary grouping of CMV strains concurrently shed by individual children. The gB variations were identified by restriction enzyme (*RsaI* and *HinfI*) digestion analysis of a 296-bp PCR product amplified from a pair of primers (gB1319/gB1604). Two typical digestion profiles were obtained by each enzyme, and the combination of profiles provided by both enzymes thus specified four possible groups of gB digest. Fifth, for CMVs belonging in the same gB group, a further typing procedure (described below) was undertaken to determine the difference between CMV strains.

The difference between CMV strains belonging to the same gB group was ascertained by the following procedures designed to evaluate the genomic variations of the entire gB gene of each CMV strain. Two pairs of restriction enzymes, *RsaI/CfoI* and *HinfI/AluI* (5 units each), digested separately a 2,680-bp PCR product that had been amplified by a pair of primers (gB0178/gB2857) [Chou, 1990]. Temperature cycling consisted of two cycles of 94°C for 3 min, 55°C for 2 min, and 72°C for 2 min, followed by 30 cycles of 94°C for 1 min, 55°C for 2 min, and 72°C for 2 min, and one cycle of 94°C for 1 min, 55°C for 2 min, and 72°C for 5 min. The digestion time was >12 hr to avoid the possibility of incomplete digestion or the presence of partial digest fragments.

A higher annealing temperature was set to enhance primer pairing stringency, and this condition consistently provided a single and unambiguous band when the PCR product was examined by electrophoresis in a 4% agarose gel. This product was confirmed further by Southern hybridization using the corresponding probe. This decreased the possibility of PCR artifacts. The digestion products were separated by electrophoresis through a 12% polyacrylamide gel, run over at least 16 hr at 70 V using Tris-Borate-EDTA buffer. An improved sensitive silver staining procedure [Bassam et al., 1991] for analysis of complex DNA profiles generated by DNA amplification was used to detect digested restriction fragments on polyacrylamide gels. To exclude the possibility of PCR artifacts, all assays were carried out twice, and the final result was based on those showing identical profiles in the two tests. For each individual child who shed CMV persistently in urine, the DNA banding pattern of his or her CMV isolate was identified and compared. Distinct CMV strains were defined by demonstrating a difference of at least one major band for either restriction enzyme digestion pair.

Blood specimens were also collected from study participants at the second and the final month of study follow-up. Serum specimens were assayed for the presence of anti-CMV IgG and IgM antibodies. In addition, to differentiate whether CMV persistently shed in urine was due to systemic viremia, this study also employed a CMV antigenemia assay [van der Bij et al., 1988] and a PCR-dot blot hybridization [Shen et al., 1994] to detect virus in blood. The antigenemia assay used a pool of monoclonal antibodies directed against the lower matrix phosphoprotein (pp65) expressed by CMV-infected peripheral blood polymorphonuclear leukocytes (PMNLs).

RESULTS

At the onset of follow-up, 48.1% of this cohort of 287 children, ages 3–5, in two kindergartens had acquired CMV infection, identified by the presence of anti-CMV IgG antibody. During the follow-up period, among 148 CMV-sero-negative children at the study outset, only three children acquired primary CMV infection. This was identified by seroconversion of anti-CMV IgG antibody, resulting in a cumulative incidence of primary CMV infection of 2% in a 9-month period. Anti-CMV IgM antibody was not detected in blood from any children, except one of those three who acquired primary infection at the study outset or end.

Of the 139 CMV-infected children, 40(28.8%) had persistent CMV viruria, identified by cytopathologic effect and confirmed by PCR-dot hybridization, lasting for at least 3 months during the 9-month follow-up, and 67(48.2%) had intermittent CMV viruria. CMV was never isolated in urine from 32 CMV-infected children.

CMV isolates from 32 persistent shedders selected at random on the same occasion (the urine specimens collected in the 6th month of follow-up) were then subjected to grouping and typing (strain differentiation) to define the infection source. On the basis of the strain classification criteria defined previously [Chou and Dennison, 1991], restriction digest profiles of the PCR products clearly showed that CMV strains shed by 20 of the children belonged to Group I, and the strains shed by the other 12 children belonged to Group III (data not shown). No mixed-group strain was found among these isolates.

The comparison of strain variation of the entire CMV gB-encoding region in children who had shed virus persistently in urine is shown in Figures 1 and 2. When the amplified gene was digested with *RsaI/CfoI*, eight out of 12 children who shed Group III CMV demonstrated unique patterns of restriction enzyme digestion. Children 2 and 3 and 4 and 10 in Group III showed slightly different patterns (Fig. 1). Further digestion by enzymes *HinfI/AluI* distinguished digestion profiles of CMVs shed by children 2 and 3, but did not differentiate CMV shed by children 4 and 10. However, these two children were at different kindergartens, and contact between them was unlikely. These observations indi-

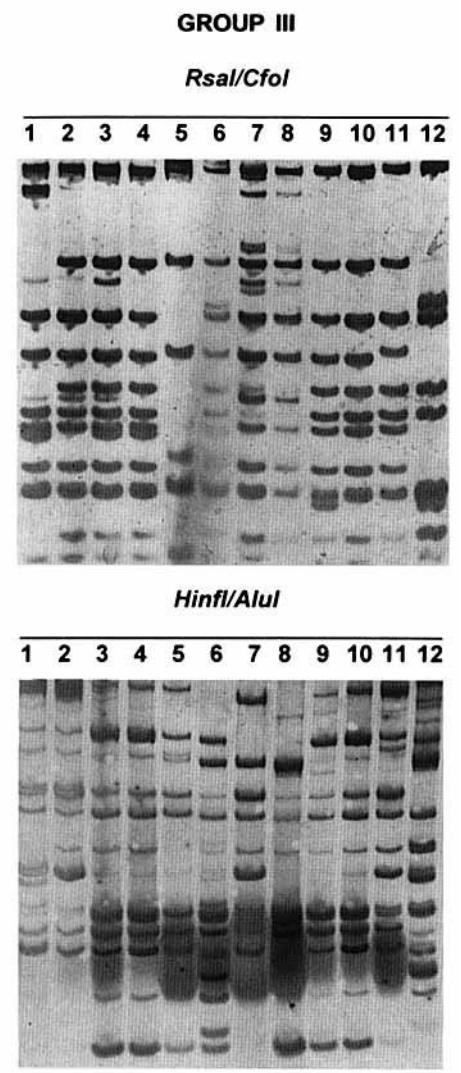

Fig. 1. The glycoprotein B (gB) gene of CMV amplified from HEL cultures inoculated with urine specimens of 12 children who shed Group III CMV was digested with combinations of restriction enzymes (*RsaI/CfoI* and *HinfI/AluI*). Each lane is the digested gB DNA of a CMV isolate from one individual child, as indicated by number. (Note: Children 3,6,7,8,10,11,12 were from kindergarten A; children 1,2,4,5,9 were from kindergarten B.)

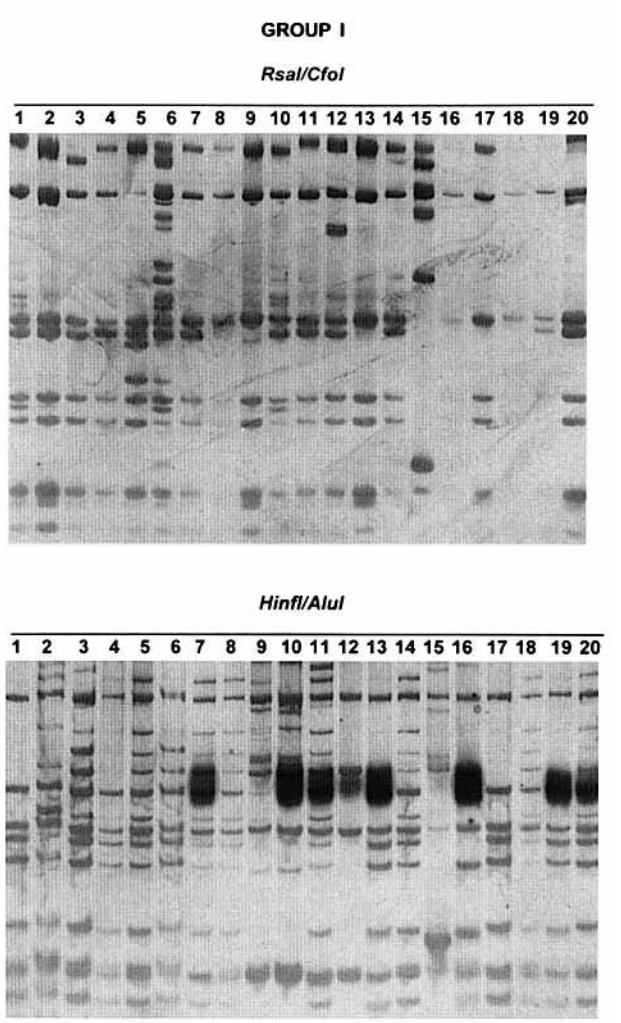

Fig. 2. The glycoprotein (gB) gene of CMV amplified from HEL cultures inoculated with urine specimens of 20 children who shed Group I CMV was digested with combination of restriction enzymes (*RsaI/CfoI* and *HinfI/AluI*). Each lane is the digested gB DNA of a CMV isolate from one individual child, as indicated by number. (Note: Children 1,5,6,7,16,19,20 were from kindergarten A; children 2,3,4,8,9,10,11,12,13,14,15,17,18 were from kindergarten B.)

cate that these 12 children shed CMV that originated from different sources.

In contrast, three set of children (8/16/18, 9/13, and 3/4/7/11/14/20) who shed Group I CMV had similar (but not identical) fragment patterns when the gB gene was digested by *RsaI/CfoI*, whereas the fragment patterns of the other nine children were visibly different (Fig. 2). Further examination of *HinfI/AluI* digestion profiles showed that there was a visible difference between children 9 and 13. In addition, the pattern of child 16 differed from that of children 8 and 18. The latter two children attended different kindergartens, excluding the possibility of mutual transmission. Major differences in fragments were present when CMV DNA was compared among children 4, 11, and others. Children 4 and 11 thus had different CMVs from each other and from the CMVs of 3, 7, 14, and 20. The CMVs from children 7 and 20 or 3 and 14 could not be differentiated. Therefore, except for four children (at the same kindergartens), the CMVs (both group I and III) shed persistently by most (28) children in these two kindergartens were not due to intrakindergarten transmission. Rather, persistent reactivation of endogenous CMV is the most plausible explanation.

The results of both the antigenemia and PCR assays showed that CMV was not found in the blood of any of these children at the onset or at the end of the study follow-up.

DISCUSSION

The answer to the important question of how to differentiate between different CMV strains remains in-

triguing. The conventional usage of cross-neutralization tests that detect antigenic variations in CMV strains is not generally acceptable. Antigens do vary among CMV isolates, but these variations are not sufficiently large to warrant the designation of different types (strains) of CMV [Ho, 1991]. The analysis of DNA difference in CMV isolates, such as restriction endonuclease digestion patterns or nucleic acid hybridization, is therefore the most common method. Such methods do not provide information that is appropriate for assigning different serotypes. However, the major concern is not related to serotyping but is to define the CMV infection source. Therefore, these CMV DNA methods are sufficiently sensitive to meet this goal.

Recent developments [Chou, 1990] in differentiation of CMV strains by restriction analysis or direct sequencing of DNA segments amplified by PCR from clinical specimens continue to progress. In the present study, restriction enzyme digestion of a PCR product containing the entire gB nucleotide sequence was used to demonstrate similarity/difference among virus isolates, and enormous genomic variety was found. Given that the rates of mutation during DNA replication in DNA viruses, such as CMV, are very low (which is believed to be associated with replication enzyme proofreading and/or DNA repair mechanisms), the possibility of the same CMV being acquired from the same infection source but experiencing mutation during a short time to cause different restriction enzyme digestion patterns in different individuals may be excluded. In addition, since the gB sequence (2 kb long) examined constitutes only a very small proportion of the entire CMV 240kb genome, the possibility cannot be excluded that those isolates demonstrating similar gB-digestion patterns in this study were actually different in other genomic regions. Consequently, the genomic differences among CMV strains shed by the children in kindergartens might be underestimated, and therefore the conclusion implying that most children persistently or intermittently shed endogenous CMV strains would be conservative.

The findings demonstrate that CMV shedding in urine was very common in children ages 3–5 in kindergartens, and >70% of children shed virus during a 9-month period. However, the data do not support the view that this shedding was caused by virus transmitted among these children. As well as the finding that the rate of primary CMV infection was very low (only 2%) and only one child showed detectable IgM antibody, the results show that almost all children tested had their own particular CMV strain. Thus persistent or intermittent reactivation of endogenous CMV is the most plausible source of CMV shedding in the children. Because of the absence of CMV in blood identified by negative antigenemia and PCR-dot hybridization, this study excluded the possibility that viruria observed was secondary to systemic infection.

Although it has not often been detected, simultaneous presence of multiple strains of CMV has been identified in immunocompromised patients such as organ transplant recipients [Chou, 1989] and patients with AIDS]Drew et al., 1984], in a heterosexual man with CMV mononucleosis [McFarlane and Koment, 1986], and in women attending clinics for sexually transmitted diseases [Chandler et al., 1987]. Thus the major concern of our result is that the children could have been infected with more than one strain of CMV, but only the dominant strain at the time of the sampling was being detected. The consistency of CMV gB groups shed by individual children on different occasions over the study period was examined to determine whether there was any variation (a possible indication of infection with multiple strains). It was found that the urinary CMV gB groups shed by almost all children remained unchanged during the study period (except in one boy, who shed strains that changed). Plaque purification is now in progress to obtain pure strains to address this issue.

The finding of persistent reactivation of endogenous CMV among kindergarten children is comparable to that reported in Sweden [Grillner and Strangert, 1988]. An interesting similarity between Taiwan and North European countries is a relatively high rate (>70%) of seropositivity in the adult population of child-bearing age. Presumably, these high rates contribute to a strong possibility of transmission from parents to children during the early stages of life, either congenitally or during early infancy, and result in ~50–60% of infection rate observed among toddlers and young children in Taiwan. Congenital CMV infections are characterized frequently by chronic excretion of the virus over several years [Stagno et al., 1975]. Also, natal CMV infections may be manifested by long-term viral excretion [Stagno et al., 1975]. In Taiwan, an infected birth canal and breast milk are the major modes of CMV transmission [Shen et al., 1992], underlying the possibility of early infection in children and subsequent long-term reactivation. It is considered that this early infection of children may be a critical factor in determining the relative importance of endogenous reactivation versus exogenous reinfection, explaining the discrepancies among different studies. Most congenitally infected children will not stop shedding until the age of around 6 [Alford and Britt, 1990]. Our study children were ≤5 yr and presumably acquired their CMV infection either congenitally or at an early age. Therefore, in this age group an endogenous source and endogenous strain(s) of CMV are more important and more dominant. When they grow up, infection sources from playmates might take the central role, as reported in an older population [Shen et al., 1993a]. We are continuing to follow these children to test this hypothesis.

REFERENCES

Adler SP (1985): The molecular epidemiology of cytomegalovirus transmission among children attending a day care center. Journal of Infectious Diseases 152:760–768.

Alford CA, Britt WJ (1990): Cytomegalovirus. In Fields BN, Knipe DM (eds): "Fields Virology." New York: Raven Press, pp 1981–2011.

Bassam BJ, Caetano-Anolles G, Gresshoff PM (1991): Fast and sensi-

tive silver staining of DNA in polyacrylamide gels. Analytical Biochemistry 196:80–83 [published erratum appears in Analytical Biochemistry 1991, 198:217].

Chandler SH, Handsfield HH, McDougall JK (1987). Isolation of multiple strains of cytomegalovirus from women attending a clinic for sexually transmitted diseases. Journal of Infectious Diseases 155: 655–660.

Chou S (1989): Reactivation and recombination of multiple cytomegalovirus strains from individual organ donors. Journal of Infectious Diseases 160:11–15.

Chou SW (1990): Differentiation of cytomegalovirus strains by restriction analysis of DNA sequences amplified from clinical specimens. Journal of Infectious Diseases 162:738–742.

Chou SW, Dennison KM (1991): Analysis of interstrain variation in cytomegalovirus glycoprotein B sequences encoding neutralization-related epitopes. Journal of Infectious Diseases 163:1229–1234.

Drew WL, Sweet ES, Miner RC, Mocarski ES (1984): Multiple infections by cytomegalovirus in patients with acquired immunodeficiency syndrome: Documentation by Southern blot hybridization. Journal of Infectious Diseases 150:952–953.

Grillner L, Strangert K (1988): A prospective molecular epidemiological study of cytomegalovirus infections in two day care centers in Sweden: No evidence for horizontal transmission within the centers. Journal of Infectious Diseases 157:1080–1083.

Grundy JE (1990): Virologic and pathogenetic aspects of cytomegalovirus infection. Review of Infectious Diseases 12:s711–s719.

Hirt B (1967): Selective extraction of polyoma DNA from infected mouse cell cultures. Journal of Molecular Biology 26:365–369.

Ho M (1991): "Cytomegalovirus, Biology and Infection." New York: Plenum.

McFarlane ES, Koment RW (1986). Use of restriction endonuclease digestion to analyze strains of human cytomegalovirus isolated concurrently from an immunocompetent heterosexual man. Journal of Infectious Diseases 154:167–168.

Shen CY, Chang WW, Chang SF, Chao MF, Huang ES, Wu CW (1992): Seroepidemiology of cytomegalovirus infection among children between the ages of 4 and 12 years in Taiwan. Journal of Medical Virology 37:72–75.

Shen CY, Chang WW, Chang SF, Huang ES, Wu CW (1993a): Cytomegalovirus transmission in special-care centers for mentally retarded children. Pediatrics 91:79–82.

Shen CY, Chang SF, Yang SL, Huang ES, Wu CW (1993b): Urinary cytomegalovirus shedding profile in children with subclinical infection. Lancet 342:1432. [published erratum appears in Lancet 1994; 343:64].

Shen CY, Chang SF, Lin HJ, Ho HN, Yeh TS, Yang SL, Huang ES, Wu CW (1994): Cervical cytomegalovirus infection in prostitutes and in women attending a sexually transmitted disease clinic. Journal of Medical Virology 43:362–366.

Stagno S, Reynolds DW, Tsiantos A, Fuccillo DA, Long W, Alford CA (1975): Comparative serial viral and serologic studies of symptomatic and subclinical congenitally and natally acquired cytomegalovirus infections. Journal of Infectious Diseases 132:568–577.

van der Bij W, Torensma R, van Son WJ, Anema J, Schirm J, Tegzess AM, The TM (1988): Rapid immunodiagnosis of active cytomegalovirus infection by monoclonal antibody staining of blood leukocytes. Journal of Medical Virology 25:179–188.